DISSERTATIONES SCHOLA DOCTORALIS SCIENTIAE CIRCUMIECTALIS,
ALIMENTARIAE, BIOLOGICAE. UNIVERSITATIS HELSINKIENSIS

19/2017

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Antibiotic Resistance in Agroecosystems

DIVISION OF MICROBIOLOGY AND BIOTECHNOLOGY
DEPARTMENT OF FOOD AND ENVIRONMENTAL SCIENCES
FACULTY OF AGRICULTURE AND FORESTRY
DOCTORAL PROGRAMME IN MICROBIOLOGY AND BIOTECHNOLOGY
UNIVERSITY OF HELSINKI

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ANTIBIOTIC RESISTANCE IN AGROECOSYSTEMS

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public examination at Biocenter 2, lecture room 1041, Viikinkaari 5, on October 13th 2017, at 12 o'clock noon.

Helsinki 2017

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ISBN 978-951-51-3671-8 (Paperback)
ISBN 978-951-51-3672-5 (PDF)
ISSN 2342-5423 (Print)
ISSN 2342-5431 (Online)

Hansaprint
Helsinki 2017

Suomalaiselle maataloudelle

To Finnish agriculture

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Ruuskanen, M.; Muurinen, J.; Meierjohan, A.; Pärnänen, K.; Tamminen, M.; Lyra, C.; Kronberg, L.; Virta, M., Fertilizing with animal manure disseminates antibiotic resistance genes to the farm environment. *Journal of Environmental Quality* 2016**,** *45* (2), 488–493.

II Muurinen, J.; Stedtfeld, R.; Karkman, A.; Pärnänen, K.; Tiedje, J.; Virta, M., Influence of manure application on the environmental resistome under Finnish agricultural practice with restricted antibiotic use. *Environmental Science & Technology* 2017, *51* (11), 5989–5999.

III Muurinen, J.; Pasupulate, A.; Lappalainen, J.; Virta, M, Field-compatible protocol for bioluminescent bioreporters without pipetting steps. Submitted.

The publications are referred to in the text by their roman numerals.

AUTHOR CONTRIBUTION

I Johanna Muurinen designed the work together with Marko Virta and participated in the experimental work, with the exception of the HPLC analyses. She participated in interpreting the results and wrote the manuscript together with Matti Ruuskanen.

II Johanna Muurinen designed the work together with Marko Virta and did most of the experimental work except for run of samples with the qPCR array. She analyzed the results and wrote the manuscript.

III Johanna Muurinen participated in the design of the work and experimental work. She analyzed the results together with the co-authors and wrote the manuscript.

ABBREVIATIONS

APF	Animal protein factor
ARG	Antibiotic resistance gene
DNA	Deoxyribonucleic acid
ESBL	Extended-spectrum β lactamase
EU	European Union
FAO	United Nations Food and Agriculture Organization
HGT	Horizontal gene transfer
HPLC	High performance liquid chromatography
IC	Induction coefficient
LMM	Linear mixed model
MGE	Mobile genetic element
MRSA	Methicillin resistant *Staphylococcus aureus*
OIE	World Organization for Animal Health
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
RLU	Relative light unit
UN	United Nations
US	United States of America
USDA	United States Department of Agriculture
WHO	World Health Organization

ABSTRACT

Agricultural antibiotic use is considered to foster the development and spread of resistant pathogens threatening human health and thus it is suggested that the use of antibiotics in production animals should be limited. Unlike in many big countries in the world, Finnish animal production uses antibiotics predominantly for treating bacterial infections. Nonetheless, the circulation of antibiotic resistance genes through intersecting ecosystems, such as production animal farms, might have a considerable role in transferring resistance genes from environmental bacteria to human or animal associated bacteria. Agroecosystems are unique settings where bacteria originating from animals and the environment are constantly mixed due to land application of manure and ingestion of harvested forage by production animals.

In this work it was clarified if genes related to antibiotic resistance and transfer disseminate from farms to the environment due to manure fertilization, if the abundance of these genes is affected by winter storage of manure and if resistance gene abundances are elevated in soils at crop harvesting time, and thus potentially cycled into the animal gut via forage. A field-compatible protocol for detecting antibiotic concentrations sensed by bacteria in different samples was also established.

Manure had the highest abundance of genes related to antibiotic resistance and transfer, and the abundance increased during storage. The genes abundant in manure disseminated to soil when manure was applied; however, at the harvesting time the soil resistome resembled the resistome of unfertilized soil. Antibiotic resistance genes were also detected in ditch water but most of them were undetected in manure, suggesting that genes in manure were not spreading to receiving waters. The results propose that manure fertilization does not inevitably generate a risk of disseminating antibiotic resistance and that agricultural practices largely determine whether or not the use of antibiotics in production animals contaminates the agricultural environment with resistance determinants. The presented field-use suitable assay can be used for samples with minor matrix-effects, e.g. surface waters. With further method development, the protocol could help in progressing from detection of antibiotics to the evaluation of their ecological effects in the studied environment.

TIIVISTELMÄ

Antibioottien holtittoman käytön takia yleistynyt bakteerien antibioottiresistenssi uhkaa koko ihmiskuntaa. Ongelman ratkaisemiseksi on ehdotettu antibioottien käytön vähentämistä eläintuotannossa, sillä on osoitettu, että resistenttien bakteerien aiheuttamien infektioiden ja antibioottien ei-terapeuttisen käytön välillä on yhteys. Suomessa antibiootteja käytetään vain vähän ja vain lääkkeenä. Tuotantoeläinten lantaa käytetään lannoitteena kaikkialla maailmassa. Lannoitetuilta pelloilta korjataan satoa, joka syötetään eläimille rehuna. Tämä johtaa suolistobakteerien ja maaperäbakteerien jatkuvaan sekoittumiseen, ja molempien ympäristöjen bakteerit kantavat resistenssigeenejä.

Tässä työssä selvitettiin, levittääkö suomalainen kotieläintuotanto antibioottiresistenssiä ympäristöön. Näytteitä otettiin tuoreesta lannasta, talven yli varastoidusta lannasta sekä maasta ennen lannoitusta ja lannoituksen jälkeen rehun korjuuseen asti. Lisäksi tutkittiin salaojavesiä. Työssä kehitettiin myös kenttäkäyttöinen menetelmä, joka mittaa bakteerien havaitseman (biosaatavan) osuuden antibioottijäämistä, mitkä voisivat levittää resistenssiä.

Bakteerien antibioottiresistenssigeenit levisivät maahan lannan levityksen seurauksena. Leviämistä vesistöön ei havaittu. Vaikka resistenssigeenit levisivät maahan, laski niiden lukumäärä ja suhteellinen osuus selvästi jo kuuden viikon kuluttua lannoituksesta. Lisäksi ennen lannoitusta otettujen maanäytteiden resistenssigeeniprofiili oli samankaltainen, kuin luonnonpuistoilla. Työssä huomattiin myös, että bakteerien antibioottiresistenssigeenit rikastuvat lannan talvivarastoinnin aikana kaikilla tiloilla. Tämä tarkoittaa, että lannan varastointi lisää ympäristöön joutuvien antibioottiresistenssigeenien määrää. Vaikka tässä työssä ei löydetty antibioottijäämiä, niitä on kuitenkin maailmanlaajuisesti löydetty maatalousympäristöistä. Kehitetty menetelmä bakteereihin vaikuttavien antibioottijäämien mittaamiseen sopii sellaisenaan esimerkiksi valuntaa vastaanottavien vesien tutkimiseen, mutta maanäytteiden osalta menetelmää tulisi kehittää.

1 INTRODUCTION

1.1 HISTORY OF ANTIBIOTICS AND THEIR USE IN ANIMAL HUSBANDRY

The discovery of antibiotics in the 20th century made deadly infections curable. The wonder drugs generated welfare, economic growth and allowed the development of the modern carefree lifestyle. However, the overuse of antibiotics has accelerated the development of bacterial antibiotic resistance resulting in an emergence of multidrug-resistant pathogens (Levy and Marshall, 2004). According to the most pessimistic views, humankind might be entering a "post-antibiotic era" where common conditions such as strep throat or infected wounds could kill again (Kåhrström, 2013).

Scientists Paul Ehrlich and Alexander Fleming established the antibiotic era with their inventions. Ehrlich discovered Salvarsan as a medicine for syphilis, which was practically incurable in the beginning of the 20th century. Ehrlich's systematic screening of chemicals for potential drugs was later used by Bayer chemists in the discovery of sulfa drugs, the first antibacterial chemicals on market, in the early 1920s (Aminov, 2010). Even today, sulfonamides are widely used in production animals (European medicines agency 2015).

The story of the life-saving wonder drug penicillin began when Fleming (1929) discovered that *Penicillium* mold inhibits the growth of *Staphylococcus* variants. However, Fleming had difficulties with the purification and stability of the active substance, and he supplied the penicillin-producing strain to a number of chemists, hoping that somebody could overcome the difficulties (Aminov, 2010). Finally, in 1940, a group of Oxford chemists published a paper where most of the problems were resolved (Chain et al., 2005). The work by Chainet al. (2005) eventually led to mass production of penicillin during World War II at the pharmaceutical company Merck & Co., Inc., in New Jersey, US (Woodruff, 1981).

During the time when Fleming was struggling with his miracle drug, another future Nobel Prize winner, Selman Waksman, was working with soil bacteria that were antagonistic to pathogenic bacteria (Waksman and Woodruff, 1940a). He found Fleming and Chain's work inspirational and started screening compounds with similar effects from *Actinomycetes* spp. together with his student Boyd Woodruff. The pair found substances capable of inhibiting pathogenic bacteria (e.g. Waksman and Woodruff, 1940b). As an outcome of their work, Woodruff was recruited by Merck & Co. to work on fermenting processes used to produce penicillin. During World War II, penicillin was one of the major projects of the American pharmaceutical industry. Waksman had just coined the name "antibiotics", and was working together with Howard Florey, Norman Heatley and Woodruff on penicillin fermentation and isolation at Merck & Co (Woodruff, 1981). Later it turned out that the fermentation process these scientists advanced spurred the growth promotion use of antibiotics.

Before the time when fermentation processes produced antibiotics for the massive need created by World War II, Merck & Co. had announced the discovery of vitamin B12—finally a cure for pernicious anemia. However, a major problem was

that the vitamin was extracted from the liver, and thus the process could not generate a sufficient yield to fill the growing demand (Woodruff, 1981). Researchers working at Merck & Co. had become experts on fermentation and soon discovered that vitamin B12 could be produced in the same deep-tank fermentation vats where antibiotics were produced (Wood and David, 1952).

While mass production of antibiotics and vitamin B12 were developed in pharmaceutical companies, the United States Department of Agriculture (USDA) had encouraged researchers to find food supplements that could make the production animals stay in good health and gain more weight in order to meet the huge demand for meat and eggs. Farmers were pressured with advertisements and campaigns to aim for high productivity in order to produce food for US soldiers fighting in World War II (USDA, 1940s). It was known that by feeding chickens and pigs with fodder containing animal protein, they grew faster, were healthier, and laid more eggs, but the Pearl Harbor attack had caused problems in animal protein supplies for US, since fish industry leftovers from Japan could not be utilized anymore. Therefore, researchers tried to find a replacement, the animal protein factor (AFP), an ingredient responsible for enhanced productivity and health, aiming to develop a feed supplement with this effect. As one of the results from multiple experiments on production animals, it was found that feeding chickens with dry cow manure improved productivity and decreased mortality caused by diseases (Rubin and Bird, 1946). It was suspected that this was due to the fact that the manure contained microorganisms that produced vitamin B12 and therefore B12 was assumed to have similar effects as animal protein.

Another scientist, plant physiologist Benjamin Duggar, was screening soil microorganisms for new antibiotics at Lederle Laboratories, a division of American Cyanamid, in New York. He was successful and discovered Aureomycin (Duggar, 1948). Biochemist Thomas Jukes, also working at Lederle Laboratories, discovered that the waste products from the Aureomycin fermentation process contained vitamin B12, along with the antibiotic-producing organism. Jukes began feeding trials on production animals with the fermentation remains and found that vitamin B12, possibly the APF, together with Aureomycin leftovers, gave favorable results (Stokstad and Jukes, 1950; 1951; Stokstad, et al., 1949). However, very soon it was discovered that the supplement responsible for weight gain and enhanced health was not vitamin B12, as initially hypothesized, but rather the antibiotic Aureomycin, also known as chlorotetracycline (Briggs and Beeson, 1952; Groschke and Evans, 1950). This finding led to the mass use of antibiotics as growth promoters and disease-preventing food supplements in production animals.

So began the non-therapeutic use of antibiotics in food animals, as the outcome of a coincidence, resembling Fleming's great discovery. Warnings from researchers like Rammelkamp and Maxon (1942), and Fleming himself, on antibiotic resistance were ignored. Ironically, already over fifty years ago, the editors of the New England Journal of Medicine wrote that if the correlation between bacterial resistance against antibiotics and antibiotic use will continue to be overlooked, "the physicians will find themselves back in the preantibiotic Middle Ages with the treatment of infectious diseases" (Anon., 1966).

1.1.1 THE HISTORY OF ANTIBIOTIC USE IN ANIMAL HUSBANDRY IN FINLAND

Most successful agricultural crops are grasses in many parts of Finland due to the geographic location, and thus most Finnish production animal farms are dairy farms. Agricultural antibiotic use in Finland is low on a global scale. In the surveys of the European Surveillance of Veterinary Antimicrobial Consumption Project, Finland has been among the countries with the lowest veterinary antibiotic consumption per production animal (e.g. European Medicines Agency, 2013; 2015). Over half of the veterinary antibiotics sold in Finland consist of benzylpenicillin, and over a quarter of the sales are either sulfonamides or tetracyclines (European Medicines Agency, 2013; 2015). The use of fluoroquinolones and extended-spectrum β-lactams in production animals is rare in Finland (European Medicines Agency, 2015). As in the rest of the EU, antibiotics are allowed to be used only in the medication of production animals. However, without determined professionals the situation might be different.

At the time when the mass-production of antibiotics started in pharmaceutical companies, and the USDA urged researchers to conduct feeding trials on production animals to find the APF, there was a war on Finnish territory. The population suffered famine during wars and there was a shortage of everything. After the last war, refugees from Karelia that had to be resettled accompanied the malnourished people, and finally the technical development of agriculture that had been on hold during the wars could be continued. After the wars farmers were keen to increase crop yields and kilograms of animal products. In addition, the Faculty of Agriculture and Forestry at the University of Helsinki had trained experts, who were determined to fuel food production with their knowledge (Simonen, 1944).

Finnish soils are naturally acidic and poor in phosphorus, and thus academically educated agronomists urged farmers to mix "superphosphate" (monocalcium phosphate) with manure that was still the main fertilizer before the 1950s (Simonen, 1944; Niemelä, 2004). Finnish decision makers came up with the strategy that instead of trying to fatten production animals, the aim was to feed the hungry people and their unproductive cattle in the long term with increased crop yields resulting from enhanced use of chemical fertilizers (Niemelä, 2004). The national strategy to invest in fertilizers and subsidize poor farmers so that they could buy fertilizers was very efficient, since already in 1960 Finland had overproduction in milk, butter and eggs (Niemelä, 2004). Due to that and severe eutrophication problems in inland lakes and the Baltic Sea in the 1980s, the national agricultural policy has subsequently aimed to decrease and regulate the use of fertilizers—including manure (Niemelä, 2004).

Veterinary education in Finland started in 1946 and until 1962 the students had to finalize their degree in Sweden. Before that Finnish veterinarians studied in Germany, Sweden and Denmark (Helminen, 2017). In 1948, the Finnish Ministry of Agriculture established a law that stated that antimicrobials categorized as drugs are allowed to be administered only by prescription and veterinarians are allowed to purchase their prescription drugs in stock price, but more importantly, the veterinarians cannot make profit from the drugs they sell (Helminen, 2013; Kaartinen, 2016). The purpose of this measure was to reduce the price of drugs so that the farmers could afford them (Helminen, 2013). This legislation made the market difficult for growth promoters, as it gave noticeable influence to veterinarians. It was also a step towards the prudent use of antibiotics, since now the veterinarians could prescribe the drugs according to the latest scientific knowledge and the farmers still could afford them (Helminen, 2013). Since 1964, all medicinal products needed to be

authorized by the Finnish Medicines Agency before they could be introduced to market. Although the main focus of this regulation was in the quality and safety of drugs, their efficacy and appropriate use were taken into account. Yet another reason for the failure of non-therapeutic antimicrobial use in Finland was probably that the effects of growth promotion on milking cows are not as straightforward as with chickens and pigs. Despite all this, certain antimicrobial feed additives that were not classified as drugs were used in Finland until their ban in 1996 (FINRES, 1999). Until the late 1990s, there were practicing veterinarians in Finland known to use broad-spectrum antibiotics and antibiotic combinations for the treatment of mastitis (Pyörälä, S. personal communication, March 24, 2017). Since also relatively high levels of resistance were observed in *Staphylococci* isolated from bovine mastitis samples (Myllys, et al., 1998; Pitkälä, et al., 2004), a few highly determined veterinarians involved in teaching decided that something should be done about the matter. Thus, veterinary students were taught to use narrow spectrum benzylpenicillin and avoid the use of antibiotic combinations in the treatment of mastitis (Pyörälä, S. personal communication, March 24, 2017).

In order to identify the risks arising from agricultural antibiotic use, it is important to monitor their use and resistance levels. In 1995, Denmark established the Danish programme for surveillance of antimicrobial consumption and resistance in bacteria from animals, food and humans (DANMAP) to follow the effects of withdrawal of antimicrobial growth promoters, which were totally banned later in 2000 (Cogliani, et al., 2011). Sweden was very advanced in monitoring and restricting agricultural antibiotic use. In 1986, Sweden was the first country in the world to ban the growth promotion use of antimicrobials (Cogliani et al., 2011). Swedish officials had collected data on antimicrobial use in food animals from as early as 1980, and in 2000, the Swedish system to monitor resistance in farm animals, Svarm was established (Cogliani et al., 2011). Finland followed the example of other Nordic countries later and established the Finnish surveillance system, FINRES-vet, in 2002 to monitor resistance in farm animals. However, data on food production animals from 1998 was already included in a FINRES report co-published by the Ministy of Agriculture and Forestry and the Ministry of Social Affairs and Health (FINRES, 1999). Before that, resistance in *Salmonella* had been monitored since 1983 and the antimicrobials used in production animals since 1995 (Kaartinen, 2016). In 1996, the Finnish Ministry of Agriculture and Forestry issued a report entitled “Examples on the use of antimicrobials for infectious diseases in production animals”. This report was a highly detailed guideline for the prudent use of antimicrobials in veterinary medicine and unique at that time even among the Nordic countries. In 2003, the name of the report was changed to “Guidelines for the use of antimicrobials for infectious diseases in production animals”, and after that these guidelines have been updated in 2009 and 2016 (Evira, 2016).

Sweden and Finland joined the EU in 1995, and Finland was given two years to show that use of the feed additives tylosin and spiramycin decreases the efficiency of antibiotics to keep the ban on the use of antimicrobial drugs as feed additives and to expand it to all antimicrobial feed additives (Honkanen-Buzalski, 2016). During the process of joining the EU, Finland and Sweden lobbied in the negotiations for the total ban of the non-therapeutic use of antimicrobials in food animals. Finally, in 2006, due to several findings and promotion by scientists, all antimicrobial growth promoters were prohibited in the EU (Cogliani et al., 2011, Witte, 1998). In 2008, the European Commission asked the European Medicines Agency to harmonize the surveillance programs collecting data on antimicrobial use, resulting in the

establishment of the European Surveillance of Veterinary Antimicrobial Consumption Project (Cogliani et al., 2011).

Since the first surveillance conducted in Finland, the resistance levels in bacteria isolated from food animals have been generally favorable for most antibiotics (FINRES-vet, 2004) and they have remained good until the present (FINNRES-VET 2007, FINRES-vet 2015). However, there are also issues of concern; for example, the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in swine seems to be increasing (Evira, 2015).

1.2 EMERGING PATHOGENS FROM FARM TO FORK

There is strong evidence that antibiotic use increases the occurrence of resistance (Goossens, et al., 2005; Marshall and Levy, 2011), and globally agriculture is today the largest antibiotic user (CDDEP, 2015). Modern intensive animal husbandry is heavily dependent on antimicrobials due to high animal density on farms, supporting introduction and spread of pathogens (Wegener, 2003). Outside the EU, antibiotics are used in fodder mixtures to prevent disease and increase productivity. This kind of non-therapeutic use is not allowed in the EU. However, there are differences in antibiotic use also within EU countries. For example, in certain EU countries the use of antibiotic-combinations and broad-spectrum antibiotics in treating infections is more common than in Nordic countries (European Medicines Agency, 2015). There is increasing evidence that the use of broad-spectrum antibiotics and antibiotic combinations increases the prevalence of multiresistant bacterial strains (Fitzgerald, 2012).

Pathogenic bacteria that are resistant to multiple antibiotics are frequently called “superbugs”. MRSA is perhaps the most well-known superbug. Interestingly, the zoonotic livestock-associated MRSA (LA-MRSA) arose from methicillin-susceptible *S. aureus* that originated from people (Price et al., 2012). From people the bacteria moved into livestock, and acquired resistance to methicillin and tetracycline and dispersed further among swine (Price, et al., 2012). It is speculated that the prevalent use of tetracycline in swine could have triggered the emergence of LA-MRSA (Fitzgerald, 2012).

Recently, the World Health Organization (WHO) published a priority list of antibiotic-resistant bacteria for which new antibiotics are urgently needed, with levels from critical to medium (WHO, 2017). Bacteria belonging to top priority levels (critical & high) have been found from production animal farms all over the world (Table 1). This demonstrates that agricultural antibiotic use has a considerable role in the development of emerging pathogens and the mechanisms generating these strains should be understood more deeply. For example, despite the fact that carbapenems are not allowed to be used in production animals anywhere in the world (OIE, 2015), carbapenemase producing *Escherichia coli, Acinetobacter* spp. and *Salmonella* spp. have been found in production animals (Table 1). These resistance genes are usually located in genetic islands, which often harbor multiple resistance genes, suggesting that these bacteria might be selected due to the use of other antimicrobials besides carbapenems (co-selection) (Mollenkopf, et al., 2016) or even as a result of another unknown mechanism.

Table1. Examples of WHO's list of priority pathogens (critical & high) found in food animals and farms.

Emerging pathogen(s)	Type of farm	Type of antibiotic use	Location	Reference
Acinetobacter genomospecies 15TU, carbapenem resistant	Dairy farm	Amoxicillin-clavulanate (n=1), oxytetracycline & neomycin, mastitis treatment	France	Poirel, et al., 2012
Escherichia coli, carbapenem resistant	Swine farm	Not reported	Germany	Fischer, et al., 2012
Salmonella spp. and *E. coli* isolates, carbapenem resistant	Poultry production, Swine farm	Not reported	Germany	Fischer, et al., 2013
E. coli isolates, carbapenem resistant	Swine farm, farrow-to-finish operation	"Typical production practices."	US	Mollenkopf, et al., 2016
Methicillin resistant *Staphylococcus aureus* (MRSA) vancomycin-intermediate and resistant	Skin swab from a 45-day-old swine presenting exudative epidermitis in 2012 (n=1)	Not reported	Brazil	Moreno, et al., 2016
Campylobacter jejuni, fluoroquinolone-resistant	Poultry farms, slaughterhouse, and meat	Not reported	Spain	Melero, et al., 2012
Campylobacter Spp, fluoroquinolone-resistant	Multiple swine farms	Both antibiotic free and traditional production	US	Tadesse, et al., 2011

Although slaughterhouse hygiene standards are high in developed countries, contamination of fresh meat during carcass cutting cannot be totally avoided (Alexander et al., 2010) and the transfer of resistant bacteria from food animals to humans via meat products is well established (Marshall and Levy; 2011, Witte, 2000). In the Netherlands, it was discovered that MRSA was highly prevalent in swine (de Neeling et al., 2007), and consequently, the same subtype strains were found from meat products in retail (de Boer et al., 2009). On the contrary, in Switzerland, MRSA was not found from meat, and in general, the MRSA prevalence throughout the pork production chain was low (Huber et al., 2010). Variation in strain subtypes was found

in all these studies, indicating that there might also be alternative dissemination and contamination routes. In addition to meat products, MRSA may also spread from animals to humans through farm workers (Graveland et al., 2010). Spread of resistant bacteria from farm animals to humans via physical contact was observed long ago with streptothricin resistant *E. coli*, arising from the use of streptothricin as a growth promoter in pigs (Hummel et al., 1986).

Also horizontal transfer of resistance genes may cause variation in resistant bacteria strains observed in different parts of the food production chain. Previously, the vancomycin resistance gene *vanA* was shown to disseminate in the food production chain (Klare et al., 1995a; b) embedded in conjugative plasmids (Witte, 2000). Plasmids are mobile elements that can transfer between bacterial species and are capable of carrying multiple resistance genes. The spread of resistance genes via mobile elements increases the difficulty of identifying the antibiotic responsible for increased prevalence of resistance. However, this depends on the antibiotic. For example, fluoroquinolone resistance typically arises as a result of a target site mutation, and the prevalence of fluroquinolone resistance is lower in countries with low fluoroquinolone consumption (Redgrave et al., 2014). Therefore, reducing the use of fluoroquinolones on farms could potentially decrease fluoroquinolone resistance in *Campylobacter* spp. Nevertheless, ciprofloxacin resistant *Camplylobacter* spp. has been found in swineherds in farm-slaughter pair cohorts where antimicrobials were not used at all (Tadesse, et al., 2011). This indicates that there might also be other risk factors generating evolution that produces fluoroquinolone resistance besides the use of antimicrobials alone. Long-term evolutionary experiments have shown that fluoroquinolone resistance may arise as an outcome of selection under specific conditions even in the absence of any selective pressure from antibiotics (Crozat et al., 2005).

1.3 ORIGINS AND DISSEMINATION OF ANTIBIOTIC RESISTANCE

1.3.1 SELECTION AND GENETICS

Bacterial antibiotic resistance can be either intrinsic or acquired. Intrinsically resistant bacteria have inherited the ability to resist the effect of antimicrobial agents through physical or functional features. Natural resistance against antibiotics can arise as a result of e.g. cell membrane structures preventing the antibiotic from entering into the bacterial cell, lacking the target of the antibiotic, exporting the molecule by chromosomally encoded exporter-proteins or due to production of enzymes that inactivate the drugs (Allen et al., 2010). Antibiotic concentrations that are high enough for inhibiting the growth of susceptible bacteria cause selection of resistant populations and is therefore called selection pressure. The resistance is genetically encoded. In addition to naturally existing resistant bacteria, susceptible bacteria may also become resistant to antibiotics through a genetic mutation or as a result of acquiring resistance genes from other bacteria or from the environment, which is known as horizontal gene transfer (HGT).

The presence of antibiotics creates a selective pressure for resistant bacterial strains (Andremont, 2003; Levy and Marshall, 2004; Witte, 2000). The selected resistant bacteria may disseminate their resistance genes to other bacteria via HGT. The three main mechanisms of HGT in bacteria are: (a) transformation, the uptake of DNA from the environment by a competent cell; (b) transduction, the transfer of DNA by a bacteriophage; and (c) conjugation, DNA transfer from one bacterial cell to another via physical contact (Levy and Marshall, 2004; Muniesa et al., 2013; Thomas and Nielsen, 2005). The horizontally moving genes can maintain their functionality with the help of integrons and insertion sequences that allow the expression of the transferred genes (Gaze et al., 2011; Mazel, 2006). Integrons are gene acquisition elements that collect the genes and insert them to mobile structures, such as plasmids and transposons. Together with insertion sequences having promoter activity, integrons have created mobile genetic elements (MGEs) that often have a mosaic structure: gene cassettes collected by integrons are incorporated into transposons, which can be integrated into plasmids (Liebert et al., 1999, Mazel, 2006). As a result of transposition and integration, gene cassettes may move between the chromosome and mobile elements, and thus several genes may be collected on a single MGE (Gaze, et al., 2011, Levy and Marshall, 2004, Liebert et al., 1999). Once a resistance gene is acquired on a MGE, eliminating it from the element is difficult, since other elements on the construct can offer a fitness benefit to the host even in the absence of antibiotic selection pressure (Enne et al., 2004; Lacotte et al., 2017).

The prevalence of class I integrons in *E. coli* was shown to depend on selection caused by bactericidal substances that are common in human affected settings (Díaz-Mejía et al., 2008). Hence, the selection pressure created by antibiotics does not just select for resistance traits but might also affect the horizontal transfer of genetic materials. There is also evidence that co-selection for MGEs occurs as a result of exposure to detergents, through a quaternary ammonium compound resistance gene embedded in class 1 integrons (Gaze et al., 2011). Therefore, by using one particular bactericidal substance, it is possible to cause selection pressure for resistance to unrelated antibiotics and for other substances, such as metals or disinfectants (Bednorz, et al., 2013; Gaze, et al., 2011). In addition, antibiotic exposure may increase the acquirement of foreign DNA by transformation (Prudhomme et al., 2006).

The establishment of resistance genes in MGEs can be further induced by stress mechanisms. SOS is a response mechanism in bacteria that can be triggered by e.g. the presence of single-stranded DNA (Gudas and Pardee, 1975), and such DNA damage can occur under sub-lethal concentrations of antibiotics or other toxic substances (Beaber et al. 2004; Gaze et al., 2011; Guerin, et al., 2009). The SOS response aims to repair damaged DNA by increasing mutation rate, which in turn is shown to lead to the development of new resistance mechanisms and horizontal acquisition of genetic material, including ARGs (Beaber et al., 2004 Guerin et al., 2009). The SOS response also regulates the expression of integrase making integrons very cost-efficient (Guerin et al., 2009; Lacotte et al., 2017). This explains the success of integrons in many bacterial species and supports their selection in the presence of subinhibitory antibiotic concentrations (Lacotte et al., 2017).

Interestingly, unfavorable environmental conditions can also stimulate multiple stress response mechanisms in bacteria, resulting in new resistance features due to enhanced HGT or as a consequence of mutations (Poole 2012; Crozat et al., 2005). Stress response mechanisms arising from numerous triggers might thus accelerate the

evolution of antibiotic resistance (Gillings and Stokes, 2012), in addition to the effect of selection pressures (Baquero et al., 2008).

1.3.2 ANTIBIOTICS AND RESISTANCE GENES IN THE ENVIRONMENT

Most antibiotics are compounds produced by environmental organisms, and these organisms are still screened to find new antibiotics. An immeasurable number of organisms living in soil interact with each other through molecules, which makes the soil a huge reservoir of organisms capable of producing bioactive chemicals (Allen et al., 2010; Davies and Davies, 2010; Wright, 2010). As microorganisms inhabit all ecosystems and are the oldest residents in our planet, it is understandable that the diversity of chemicals environmental bacteria have faced since the dawn of biosphere has enhanced their capability to adapt to the effects of various compounds (D'Costa, et al., 2006; Riesenfeld et al., 2004; Wright, 2010). Estimates on the origin of naturally produced antibiotics range from tens to hundreds of millions of years, and resistance genes can be expected to be just as ancient (Aminov 2010; D'Costa; et al., 2011; Allen et al., 2010). This theory is in agreement with the fact that the environment is a huge reservoir of resistance genes (D'Costa et al., 2006; Riesenfeld et al., 2004).

The environmental reservoir of resistance features could act as an important source in the development of resistant pathogens (D'Costa et al., 2006; 2011; Riesenfeld et al., 2004; Wright, 2010). Antibiotic producing strains often carry genes conferring resistance against the molecules they produce (Martin and Liras, 1989). There is evidence that certain resistance genes have transferred from antibiotic producers (*Actinomyces* spp.) to human and animal associated Gammaproteobacteria with the help of MGEs (Tamminen et al., 2012). Already over 40 years ago it was discovered that antibiotic producers in the environment harbor similar enzymes producing resistance to aminoglycosides as clinical strains (Benveniste and Davies, 1973). Genes encoding the CTX-M extended-spectrum β-lactamase were discovered in soil samples taken before their current clinical emergence (Knapp et al., 2010), and indeed, these genes probably originally belonged to environmental organisms (Humeniuk et al., 2002; Poirel, et al., 2002). These findings indicate that by using antibiotics, humans have selected bacteria that carry resistance genes, and as a result of adaptation to antibiotic use, some of these genes have been integrated in MGEs, that can further transfer the genes between bacteria strains and species (D'Costa, et al., 2011; Humeniuk et al., 2002; Perry and Wright, 2013; Tamminen, et al., 2012).

Antibiotic resistance is ancient and therefore existed ages before the antibiotic era (D'Costa et al., 2011), however, the roles of antibiotics in environment are still somewhat unclear. If the antibiotic concentrations produced by natural organisms would be measured from a soil sample, the concentrations would be much lower than those used in e.g. laboratory experiments to create selection pressure for antibiotic resistance. Soils are heterogeneous mixtures consisting of solids, liquids, and gases—in addition to the countless number of organisms. It seems likely that there are different concentration gradients of antibiotics in different soil particles due to the activity of antibiotic producers (Allen et al., 2010). From an anthropogenic viewpoint, antibiotics are often assumed to be chemical weapons microorganisms produce in order to compete for resources. However, antibiotics might have other than hostile purposes in natural pristine habitats of microbes, in contrast with chemical combat shaped by anthropogenic activities in human impacted environments (Allen et al.,

2010; Aminov, 2009). Low antibiotic concentrations have been shown to induce metabolic pathways and alter transcription patterns, and one natural role of antibiotics might be related to signaling (Aminov 2009; Davies et al., 2006; Goh et al., 2002; Linares et al., 2006).

The above described biphasic bacterial response to exposure to increasing antibiotic concentrations might explain why subinhibitory concentrations of antibiotics enrich antibiotic resistance (Andersson and Hughes, 2014; Davies, et al., 2006). Human activities have created hotspots enhancing the exchange of genetic material between the environmental microbiome and human or animal associated bacteria as a result of the effects of stressors, such as changes in physical conditions and the presence of toxic substances in low concentrations, including antibiotic traces (Gillings and Stokes, 2012; Baquero; et al., 2008). These hotspots include sewage, wastewater treatment plants, aquaculture, and production animal farms (Baquero; et al., 2008). A deeper understanding of these intersecting ecosystems would perhaps help in decreasing the dissemination of antibiotic resistance.

1.4 AGRICULTURE AS A DISSEMINATOR OF ANTIBIOTIC RESISTANCE

The majority of antibiotics worldwide are used in production animals (CDDEP, 2015). When exposed to antibiotics, the resistant bacteria in the animal microbiome are selected and genetic mutations might also occur in addition to HGT (Looft et al., 2012). The selected bacteria may also be inhabitants of the local environments of animal farms, i.e. settings where the animals are kept, manure storage silos or lagoons, agricultural soils and ditches receiving run-off or leachate water from the fields (Chee-Sanford et al. 2009). Similar to antibiotics, also metals, such as zinc oxide or copper sulfate can be used for preventing infections in animals (Bednorz, et al., 2013). Both, low concentrations of antibiotics and traces of metals may cause selection of resistant bacteria (Andersson and Hughes, 2014; Bednorz et al., 2013; Berg et al., 2010; Yazdankhah et al., 2014).

The results of global antibiotic overuse can also be seen in the farm environment (Chee-Sanford et al., 2009; Heuer et al., 2011; Wellington et al., 2013; Zhu et al., 2013). Residues of antibiotics frequently administered to production animals are typically detected at up to mg kg^{-1} levels in animal manure (Kumar et al., 2005), and trace concentrations of antibiotics are found in agricultural runoff coupled with intensive farming (Campagnolo et al., 2002). Agricultural antibiotic use and repeated land application of manure has been shown to change the agricultural soil resistome over time. In farmland soils collected from 1940 to 2008, the levels of ARGs clearly rose from the pre-antibiotic era to the present (Knapp et al., 2010). The increase was especially clear with tetracycline resistance genes, and certain genes are now more than fifteen-fold more abundant than in 1970 (Knapp et al., 2010).

The majority of the selection likely occurs in the animal gut. The animal intestinal microbiome has a high diversity and a large gene pool harboring ARGs and MGEs, which can further participate in disseminating resistance from commensal bacteria to pathogenic strains (Andremont, 2003; Chee-Sanford; et al., 2009; Mazel, 2006; Netherwood; et al., 1999). The intestinal microbial community is excreted in animal feces, which are rich in nutrients and therefore used as a fertilizer for forage crops everywhere in the world. The bacteria that are adapted to the animal gut face different conditions in the farm environment, possibly leading to stress-response

mechanisms that might enforce resistance evolution (Poole, 2012) together with the bactericidal substances common in the farm environment (Andersson and Hughes, 2014; Zhu et al., 2013).

The proposed cycle of resistance determinants in Finnish agroecosystems is presented in Figure 1: Resistant bacteria are selected in the animal gut during antibiotic treatment and excreted in fresh manure. Manure containing resistant bacteria is stored over winter and applied to crop fields during the growing season. During harvest, small soil particles sheltering soil bacteria, possibly carrying resistance genes, may be transported to animals among the forage. From the soil, the resistance determinants might leach out to ditches. In countries with an arid climate and insufficient rainfall during cropping season, the croplands are also irrigated, and such practices might cause the transfer of resistant bacteria from natural waters to forage crops. If bacteria carrying resistance genes are present in the harvested forage and animals are medicated while consuming the forage, the resistance genes originating from soil could be selected and even integrated in mobile elements (Humeniuk et al., 2002).

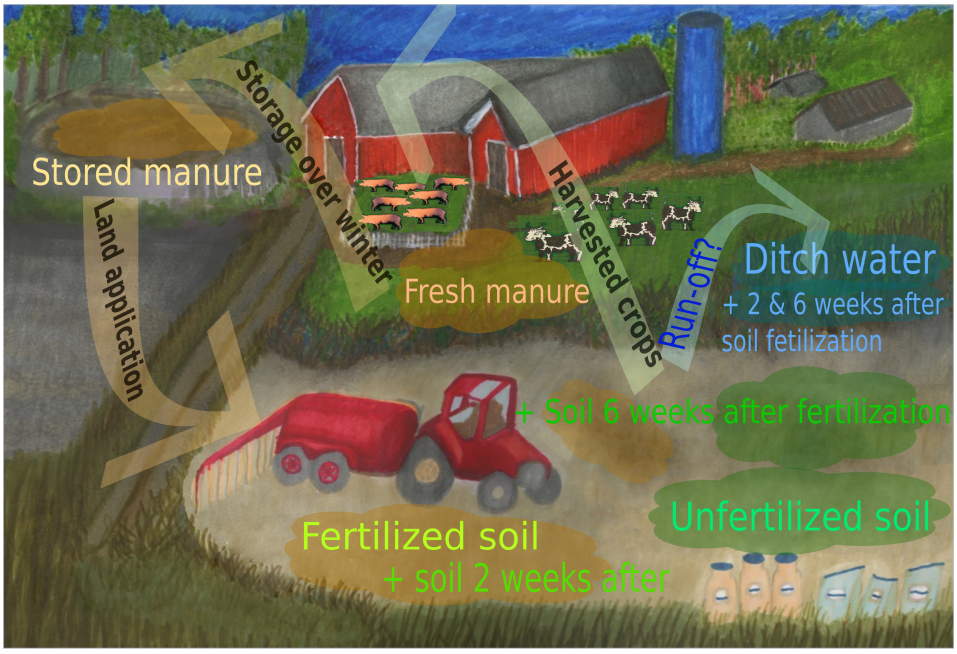


Figure 1. The proposed cycle of resistance determinants on Finnish farms due to agricultural practices (text in arrows) and samples taken from the farms (texts) in publication **II**. In publication **I**, soil 6 weeks after fertilization and ditch water samples were not taken.

Although agroecosystems possibly represent an important link from the environment to the clinic (Wright, 2010), there are only a few studies with quantitative data on the abundance of ARGs and resistant bacteria in farm environments (Garder et al., 2014; Ghosh and LaPara, 2007; Hsu et al. 2014; Luby et al., 2016; Zhu et al., 2013). These studies have been conducted in countries where the use of antibiotics is high or unmonitored, and in some studies, excessive amounts of manure have been applied to the fields. In addition, studies examining the influence of agricultural practices are

extremely rare. Among these rare studies, Lin et al. (2016) noticed that the elevation of soil ARG abundances after manure application was related to manure dosage, and Nõlvak et al. (2016) found that as the result of cattle slurry treatment, the abundance of ARGs increased in soil but decreased over time. Specifically, manure application changed the soil resistome without changing the microbial community, although excessive manure use suppressed soil microbial activity. Also, Cu and Zn in manure increased the abundance of soil ARGs (Lin, et al., 2016). The decrease of ARG abundances over time in fertilized soils was concluded to be due to the fact that the microbes carrying the genes had limited survival potential in the soil environment (Nõlvak et al., 2016).

Zhu et al. (2013) used a qPCR array to study the resistome in Chinese swine farms. Agricultural soil samples were compared to samples taken from pristine forest soil. The conclusion was that the diversity and abundance of ARGs in agricultural soil was alarming, and the unmonitored use of antibiotics and metals has really changed the farm environment. The researchers also observed co-enrichment of ARGs and transposase genes, possibly increasing the risks of transfer of ARGs from environmental bacteria to zoonotic pathogens (Zhu et al., 2013).

Intriguingly, manure alone can promote the intrinsic bacteria in soil carrying antibiotic resistance genes. This result has been obtained in two studies with functional metagenomics (Udikovic-Kolic et al., 2014) or qPCR array (Hu et al., 2016). In both studies, it was found that manure fertilization increased β-lactamase genes in soil despite the absence of antibiotic residues and β-lactamase genes in manure. Udikovic-Kolic et al. (2014) discovered that inorganic nitrogen fertilization did not elevate the abundance of soil β-lactamase genes and suspected that zinc, which exceeded average concentrations in manure, had caused selection of soil bacteria carrying these genes. Also in a study conducted in Estonia, it was found that mineral fertilization did not enhance *blaCTX-M* genes in soil, unlike cattle slurry digestate (Nõlvak et al., 2016). Supporting the finding of Udikovic-Kolic et al. (2014), it has been found that nitrogen fertilization may favor certain intrinsic bacteria, resulting in an elevated abundance of ABC-transporters and tetracycline resistance genes (Forsberg et al., 2014; Nõlvak et al., 2016). These findings demonstrate that in addition to the use of antibiotics, metals and disinfectants, alteration of the conditions in agroecosystems may also cause enrichment of antibiotic resistance due to changes in bacterial community composition.

1.5 ANTIBIOTIC RESITANCE—A CHALLENGE REQUIRING A MULTISECTORAL APPROACH

Surveys on the effects of antimicrobial use in production animals are largely based on resistant strains isolated with culturing methods from animals or foodstuffs, and Finland is not an exception (e.g. FINRES-Vet, 2007; 2015). While this work is important for public health professionals, we know that these surveillances have gaps. Denmark utilized national surveillance data in a new way by also implementing sequencing methods, and was the first country outside China that discovered the colistin resistance gene *mcr-1* from human bloodstream infection samples and indicator isolates from food-producing animals (DANMAP, 2015). However, the *mcr-1* gene was detected only because the researchers decided to look for it. Indeed, the report states that surveillance programs primarily find what they are designed to look

for and this gap should take into consideration (DANMAP, 2015). We also know that culture-dependent methods are not suitable for studying resistomes, since only a tiny proportion of all bacterial species can be cultured with current methods (e.g. Riesenfeld et al., 2004; Ward et al., 1990). The majority of existing data on antibiotic resistance is based on isolated strains and therefore it is likely that we have a somewhat biased concept on associations between antibiotic use and antibiotic resistance.

Production animal farms are recognized as one of the reactors where resistance genes from environmental bacteria may be mobilized to bacteria adapted to humans or animals with the help of selection (Baquero et al., 2008). However, public health professionals rarely consider farm environments as a source of resistance in zoonotic bacteria, despite the fact that farm environments are often included in schematic diagrams that model the movement of resistance determinants between humans, animals and environment (e.g. Davies and Davies, 2010; Wellington et al., 2013). Resistome evolution in agroecosystems can be affected by changes in environmental conditions caused by agricultural practices such as manure fertilization and antibiotic use in livestock. Accordingly, culture-independent methods are required to understand the diversity and evolution of ARGs and MGEs together with examinations of agricultural practices in order to identify potential risks.

Contamination caused by antibiotic traces has received a lot of attention. Despite the fact that with modern instrumentation it is possible to detect ultra-trace levels (sub-pg kg^{-1}) of some antibiotics, the difficulties in separating antibiotics from complex sample matrices such as soil or manure limit the screening of antibiotic concentrations in the environment. In addition, the measurements in antibiotic contamination studies are based on chemical extractions and therefore the results do not necessary correspond to the biological effect, in this case, the development or selection of antibiotic resistance (Aga et al., 2016; Song et al., 2017).

The uptake of antibiotics by crops has also been studied widely in greenhouse experiments (e.g. Dolliver, et al., 2007). While antibiotic contamination may cause harm to natural ecosystems (Halling-Sorensen et al., 2002), the risk of consumer exposure to harmful or selective antibiotic concentrations via edible crops is probably very small, since studies investigating the uptake of pharmaceuticals into plants have found concentrations in plants to be very low (Goldstein et al., 2014; Wu et al., 2014).

The current situation resembles the famous old Asian story of six blind men and an elephant (e.g. Woods, 1916) in many ways. The blind men cannot see the elephant, so they are trying to examine what is in front of them by touching. One man has the elephant's tail in his hand and says that it is a rope. Another man is touching the trunk and says that it is a snake. The third man is holding the tusk and says it is a spear, and so on. The situation in science and monitoring related to antibiotic resistance might be similar: researchers have achieved conclusions that might be somewhat correct, but the overall picture is missing, since the knowledge consists of fragmented results.

To solve this problem, the United Nations Food and Agriculture Organization (FAO) recommends to pursue more research that involves molecular and epidemiological analyses and that would provide answers to how and why resistant bacteria become incorporated into human and animal microbiomes (FAO, 2016). The organization also recommends international collaboration and a multidisciplinary One Health approach (FAO, 2016).

2 AIMS OF THE THESIS

The use of antibiotics in food animals is considered to be one of the factors behind the emergence of multidrug-resistant bacterial infections in humans. Thus, a United Nations report recommends cutting down the growth promotion use of antibiotics and the pursuit of more prudent agricultural antibiotic use (FAO, 2016). However, there is a lack of data on the environmental effects of restricted agricultural antibiotic use. Studying agroecosystems in countries such as Finland, where agricultural antibiotic use is low, would shed light on the potential risk factors generated by agricultural practices. In addition, methods for detecting antibiotic concentrations causing dissemination of resistance are needed.

In this work, I aimed to answer the following questions: do ARGs originating from animal gut disseminate to the environment (field soil and surface waters), is the abundance of ARGs affected by manure storage time, and are ARG abundances elevated in soils at crop harvesting time. My goal was also to develop a field-usable protocol for detecting antibiotic contamination sufficient to spread resistance in the environment.

In publication **I,** I wanted to see if ARGs spread into the environment on Finnish farms that use animal manure as fertilizer, despite restricted antibiotic use, and if winter storage of manure affects ARG abundance in manure. My goal was also to develop statistical methods for analyzing ARG data.

In publication **II,** I wanted see if the findings of **I** with bla_{OXA58}, *sul1*, and *tetM* hold true also with over 300 genes related to antibiotic resistance and transfer. Additionally, my aim was to discover if the abundance of ARGs in soil is elevated during harvesting time and if ARGs disseminate to receiving waters from the farms.

My goal in publication **III** was to develop a rapid protocol suitable for field use that detects bioavailable antibiotic concentrations, which could provide information on the biological effects of antibiotic traces in the environment.

3 STUDY SITES, SAMPLES AND SUMMARY OF METHODS

3.1 DESCRIPTION OF FINNISH AGRICULTURAL PRACTICES, FARMS AND SAMPLES

Farm samples were obtained from two dairy farms (T1 & T2), and two swine farms (T3 & T4), situated in southern Finland. These farms were originally selected on the basis of their differing usage of antibiotics. The use of antibiotics in Finnish animal production is restricted and limited. The veterinarians prescribe mainly benzylpenicillin for infections, and the animals are medicated individually. However, according to veterinarians, the farms T1 and T4 had suffered outbreaks during the years 2011–2013, and thus more animals were medicated with antibiotics on these farms than on the farms T2 and T3.

Manure is commonly applied to the fields in spring after the thaw and after run-off from the fields caused by melted snow has ended. Legally, the use of manure or organic soil amendments is banned from the beginning of November to the end of March in Finland. In practice, spreading of manure is allowed from mid-April to mid-October, since the legislation also takes into account the amount of rainfall and run-off risks.

Due to legislation restricting the period when manure fertilization is allowed, manure is commonly collected and stored from fall to spring (for up to ten months). Farms T1, T3, and T4 stored manure in open-air concrete silos. On farm T2, manure was stored in an open-air lagoon. In all farms, there was a constant supply of fresh manure from the cowhouse or piggery to the storage silo or lagoon through a sewer pipe. All the sampled soils were tile-drained and had been in regular agricultural use for decades, receiving manure annually. The sampled ditches receiving run-off and leachate water from the fields were chosen after consulting with farmers.

For developing the field-use suitable protocol for detection of bioavailable antibiotic contamination from environmental samples, the test samples were generated from Milli-Q water, non-homogenized milk that was purchased from a store, and agricultural soil that was obtained from the farm T4.

3.1.1 SAMPLES

The following samples were taken from all of the farms for analyses in publication **I**: fresh manure originating from multiple animals inside the animal shelter (I), stored manure from the open-air storage lagoon or storage silo (M), unfertilized soil from the field before the application of the manure (SB), fertilized soil from the field immediately after the application of the manure (SA), and fertilized soil 2 weeks after the application of the manure from the same site on the field (S2WA) (Figure 1).

For publication **II**, all of the previously described samples were taken. In addition, the soil was sampled 6 weeks after manure application (S6WA). Samples were also taken from ditches receiving run-off and leachate from the fields. Ditch water (D) samples were taken from a ditch underneath the end of a tile-drainage pipe while manure was being applied to the field. Ditch water follow-up samples were

taken from the same ditch two weeks after (D2WA) and six weeks after (D6WA) manure application (Figure 1). All samples from the farms for both studies were taken as triplicate biological replicates.

Six different tetracycline concentrations (5, 15, 25, 50, 75 & 100 ng ml^{-1} in Milli-Q water, in 10% agricultural soil slurry (in Milli-Q water), and in non-homogenized milk (Arla dairy, Hämeenlinna, Finland) were used as test samples in publication **III**. The same samples were used as controls without the addition of tetracycline. The soil used for soil test samples was obtained from the farm T4 and was tentatively described as Aquic Dystrocryept according to the Soil taxonomy system (Soil Survey Staff, 1999).

3.2 METHODS

Quantitative real-time PCR (qPCR) was used to study resistance genes in publications **I** and **II**. Soil and manure samples were sieved by hand through a 5 mm screen. After homogenization, the soil and manure samples were stored at −20°C prior to DNA extraction in publications **I** and **II**. In publication **II**, ditch water samples were filtered through a 0.2 µm pore size mixed filter and the filter was stored at −20°C prior to DNA extraction.

Stored manure samples (M) were analysed for antibiotic traces in publication **I** as follows: three biological replicate manure samples (3 g each) were extracted with methanol (30 ml), and concentrations of benzylpenicillin, cloxacillin, tetracycline, and tylosin (azithromycine) were measured. The analysis was performed with a 1260 triple quadrupole mass spectrometer (Agilent Technologies) equipped with Jetspray ESI source coupled to an Agilent 1290 series high-performance liquid chromatograph and a binary pump, a vacuum degasser, and a thermostatted column oven (30°C). A Zorbax Eclipse C18 (2.1 × 50 mm, 1.8 mm) column (Agilent Technologies) was used for separation. A detailed description of the extraction of antibiotics and analysis is provided in the supplemental material for publication **I**.

In publication **I**, the ARGs bla_{OXA58}, *sul1*, and *tetM* were selected for analysis as a result of screening with PCR (supplemental material for publication **I**). The 16S rRNA gene was quantified for normalizing ARG copy numbers to the copy number of the 16S rRNA gene in order to compare ARG profiles in different samples and avoid the bias caused by potential variability in DNA extraction efficiency. The results are thus relative abundances (for calculations, see supplemental material in publication **I**). In publication **II**, a high-throughput qPCR array with 371 primer sets was used, with eight primer sets targeting different housekeeping genes, 21 primer sets targeting MGEs, and 342 primer sets targeting ARGs. Among the housekeeping genes, 16S rRNA was the only gene detected in all samples, and therefore, ARGs and MGEs were quantified as relative to 16S rRNA gene copy number, i.e., results are presented as relative abundances (for calculations, see publication **II**).

In publications **I** and **II**, statistical analysis was performed for log10-transformed relative abundances with R (R Core Team 2013; 2015) and RStudio (Rstudio Team, 2013; 2015). In **I**, the effects of manure application, manure storage,

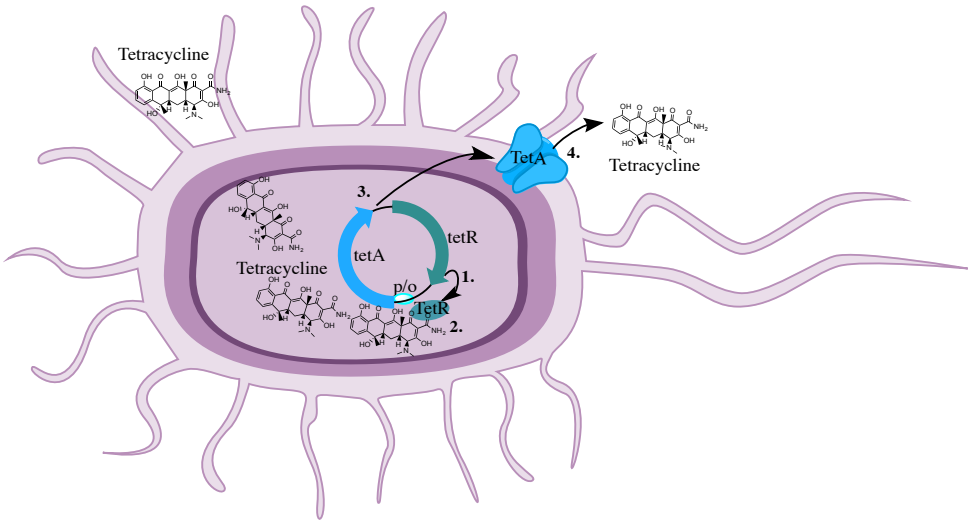
and production animal species on ARG copy numbers were analyzed statistically with linear mixed models (LMMs) and random intercepts together with fit by REML using the lme4 package (Bates et al., 2014). The significance of fixed effect fold change values were obtained by comparing the likelihood of a model with the fixed effect included against a model without the fixed effect using ANOVA- The samples compared in LMMs were: unfertilized soil (SB) to fertilized soil 2-week follow-up (S2WA) for a "manure application" model and fresh manure (I) to stored manure (M) for a "manure storage" model (Table 2). In publication **II**, the effect of environmental variables on the relative abundances of targeted ARGs was modeled with permutational multivariate analysis of variance using the vegan package (Oksanen, et al., 2016). Student's *t*-test for paired samples and false discovery rate control (Glickman et al., 2014) were used to test for the significance of fold changes in ARG and MGE abundances, calculated according to Wang et al. (2014). The network analysis in publication **II** was built with Gephi version 0.9.1. (Bastian et al., 2009) for ARG and MGE pairs with significant Spearman correlation of $\rho > 0.8$. The p-values were adjusted with false discovery rate control.

In publication **III**, to generate a protocol for detecting tetracycline contamination in the field, cleanroom processed and manufactured inert polyester swabs (Small Alpha® Swabs Texwipe, North Carolina, US were utilized together with a recombinant *E. coli* K-12 (pTetLux1) strain that produces an increasing luminescence light signal with increasing tetracycline concentrations (Korpela et al., 1998) (Figure 2). A naturally occurring resistance mechanism and live bacterial cells were utilized so that the protocol would detect antibiotic concentrations that could foster the development of resistance. To test if the protocol distinguishes different tetracycline concentrations, the swabs were first dipped in rehydrated bioreporter cells and then the same swabs were dipped in the produced test sample or control sample, following incubation for 120 minutes at 37˚C (Figure 3 in publication **III**). The luminescence was read at 490 nm using the BioOrbit 1253 (BioOrbit, Turku, Finland) and Victor 1420 (EG & G Wallac, Turku, Finland) luminometers. The whole swab assay procedure was conducted independently for both luminometers. Induction coefficients (IC) were calculated as the in vivo luminescence ratio between tetracycline-containing test samples and control samples using the following formula:

$$IC = \frac{\text{Luminescence of the test sample (RLU)}}{\text{Luminescence of the control sample (RLU)}}, \quad (1)$$

where RLU is luminescence as relative light (emission) units. Reproducibility is critical for the suitability of the protocol in field use in order to be able to differentiate contaminated samples from uncontaminated ones. To evaluate the reproducibility of swabs, the assay procedure was repeated as ten replicates each for 100 ng ml^{-1} tetracycline test samples and control samples (0 ng ml^{-1} tetracycline). Luminescence was measured with BioOrbit 1253 after 120 minutes and 180 minutes of incubation. Standard deviations were calculated from sample RLU values.

A) Natrural plasmid giving resitance to tetracycline



B) Engineered plasmid encoding protein producing light in the precence of tetracycline

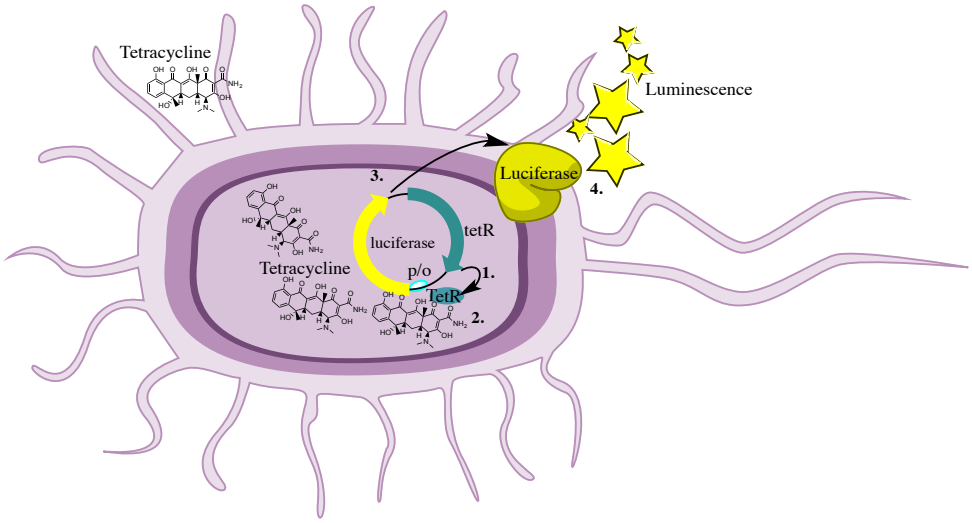


Figure 2. (A) A bacterium with a naturally occurring plasmid that confers resistance to tetracycline. (B) The used bioreporter plasmid encodes a signal-producing protein. 1: Gene *tetR* encodes the repressor protein TetR. 2: Tetracycline binds to TetR causing a conformational change and releasing TetR from the promoter/operator region (p/o). 3: (A): This allows the transcription of the resistance gene *tetA* and synthesis of the tetracycline efflux pump TetA. 3(B): This, in turn, allows the transcription of the reporter gene and synthesis of the reporter protein, luciferase. 4(A): The protein TetA pumps tetracycline molecules out of the cell, producing resistance to tetracycline. 4(B): The reporter protein luciferase produces luminescence (light signal). The figure is obtained from Aga et al. (2016).

4 RESULTS AND DISCUSSION

4.1 CHEMICAL ANALYSIS OF ANTIBIOTICS (I)

The antibiotics benzylpenicillin, cloxacillin, tetracycline, and tylosin (azithromycine) were not detected in stored manure samples (M). For this reason, they were not analyzed from manure-fertilized soils.

4.2 CHARACTERIZATION OF RESISTOME IN SOIL, MANURE AND DITCH (I, II)

To investigate the effects of restricted antibiotic use on the resistome in the farm environment, I followed four Finnish production animal farms during the cropping seasons 2013 and 2014. To examine the effect of winter manure storage on ARG abundances, samples were taken from fresh and stored manure from both years. Soils and surface (ditch) waters were sampled before and after manure application to the fields.

The relative abundances of bla_{OXA58}, *sul1*, and *tetM* were calculated by normalizing their measured copy numbers with the copy number of the 16S rRNA gene in publication **I**. All these genes were detected in all of the farms and sample types; however, *tetM* was not detected in unfertilized soil on any farm, and bla_{OXA58} was not found in manure on the farm T3 (Figure 1 in publication **I**). The qPCR array I publication **II** used 363 primer sets (assays) for detecting ARGs and MGEs and the 16S rRNA gene for normalization of gene copy numbers. In total, 182 out of 363 ARG and MGE qPCR assays were positive in one or more samples. Out of the positive assays, 161 targeted ARGs and 21 MGEs. Fresh manure had the highest diversity and abundances of ARGs and MGEs, followed by stored manure and manure-fertilized soils (Figure 3). The number of positive assays and ARG abundance decreased in fertilized soils over time (Figure 3). Unfertilized soil and ditch water samples had the lowest diversity and relative abundances of ARGs and MGEs (Figure 3).

Network analysis revealed that the detected ARGs co-occurred with MGEs (Figure 4). In agricultural farm bacteria, ARGs and MGEs of animal and environmental origin are mixed and mingled through land application of manure and forage ingestion by production animals. The circulation of bacteria and genetic material through these intersecting ecosystems might have a considerable role in transferring intrinsic environmental resistance genes to pathogenic bacteria, possibly with the support of MGEs (Baquero et al., 2008; Marshall and Levy, 2011; Witte, 2000). Animal husbandry has been shown to change and increase ARG abundances in the farm environment (Knapp et al., 2010), which is supported by these results, as a noticeable number of co-occurring ARGs and MGEs were detected.

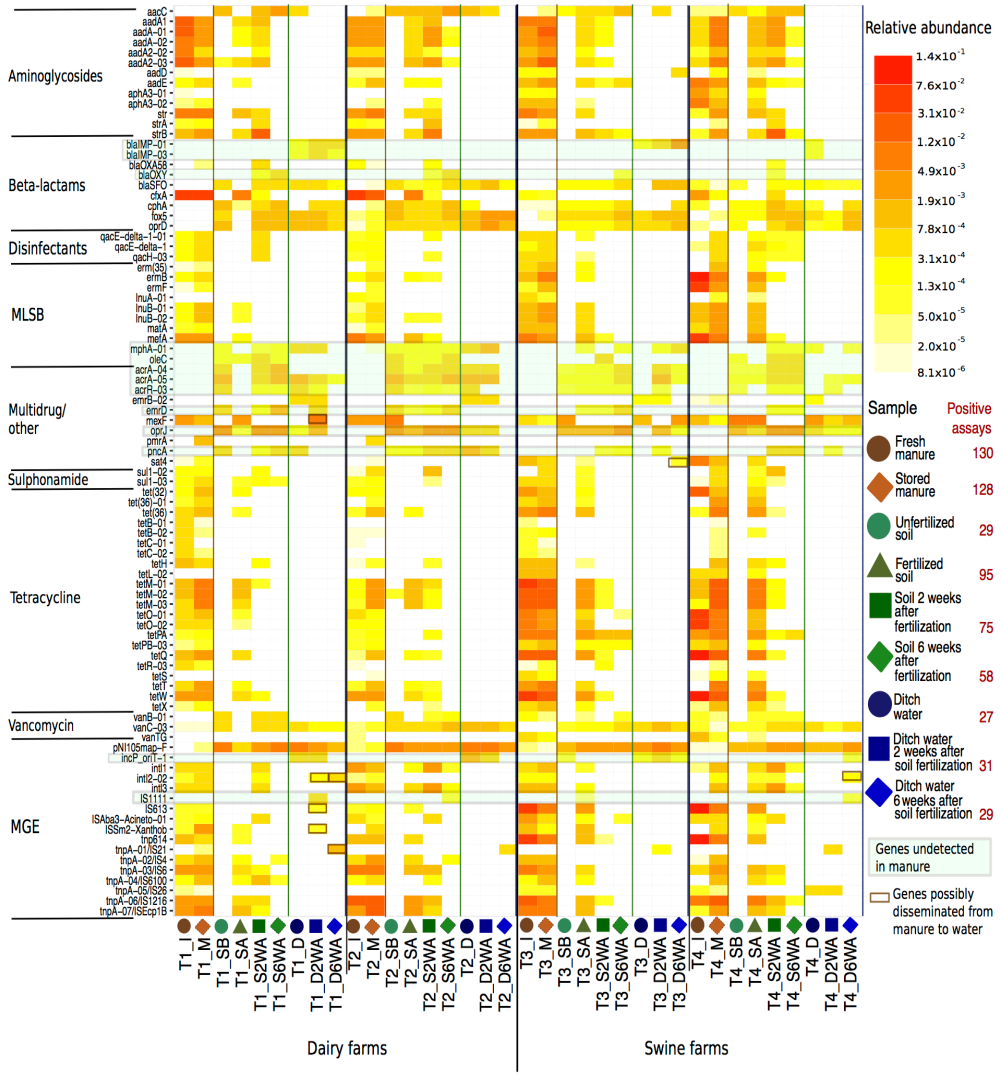


Figure 3. Heat map showing 89 genes with the highest relative abundance in samples collected from different farms. Each column is labeled with the sample code and symbol. Each row represents results from a separate primer set (assay) targeting a gene displayed on the y-axis. The assays are grouped according to the antibiotic group to which the genes confer resistance. MLSB is an abbreviation for macrolide−lincosamide−streptogramin B resistance and MGE for mobile genetic element. Dairy farms are represented by T1 & T2 and swine farms by T3 & T4. The genes undetected in manure are highlighted with faint green, and genes detected manure, soil and in ditch water after soil fertilization are circled with brown.

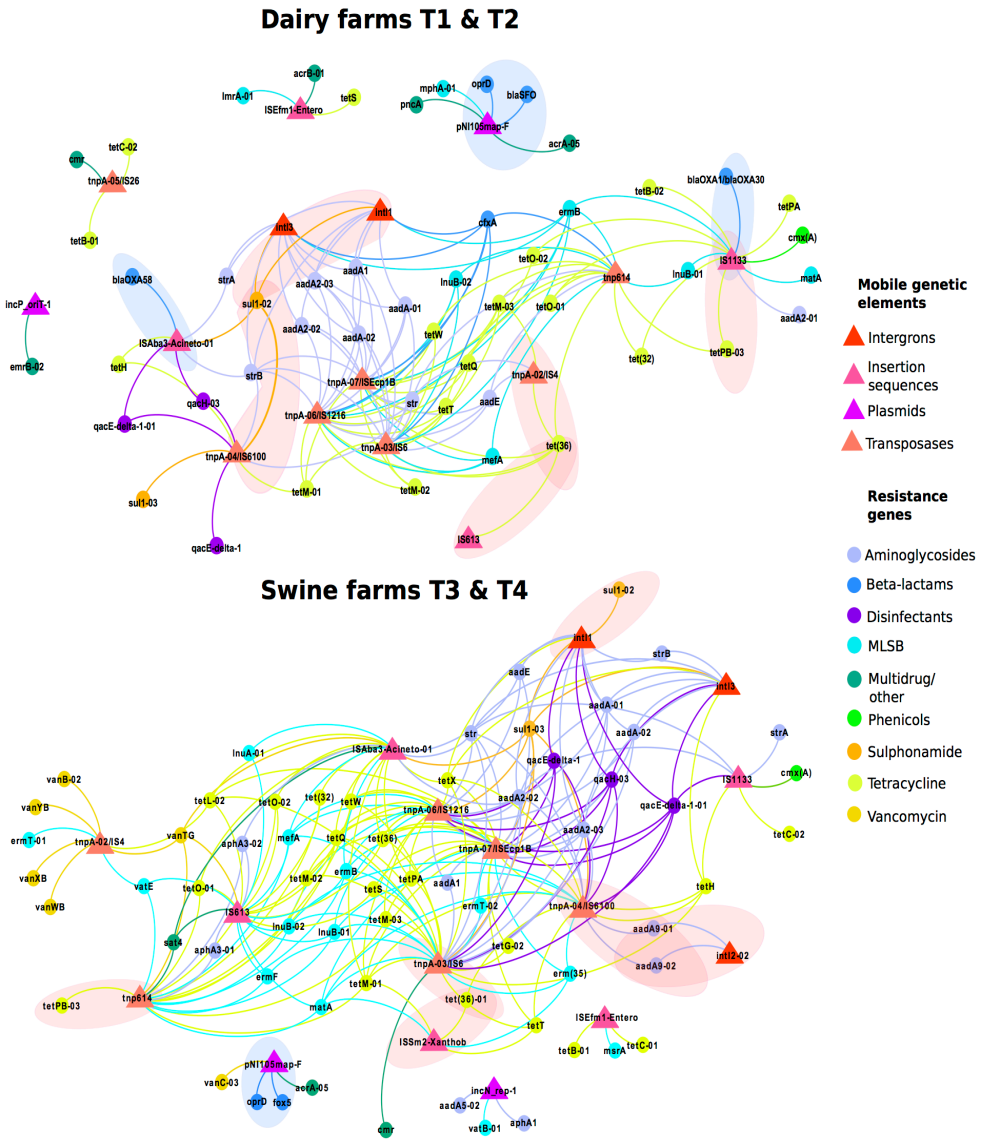


Figure 4. Network analysis revealed co-occurrence patterns between antibiotic resistance genes and mobile genetic elements (MGEs) with significant Spearman correlation > 0.8 across all samples in the two different farm types. MGE nodes are represented by triangles, and circular resistance gene nodes are colored according to the antibiotic to which they confer resistance. Edges between resistance gene nodes and MGE nodes have the color of the resistance gene node. Nodes have equal sizes, edges have equal weights, and the distance between the nodes is irrelevant. The four ARG-MGE pairs displaying highest enrichment in stored manure are highlighted with red, and β-lactamase genes co-occurring with MGEs with blue.

Manure, unfertilized soil and ditch water had their characteristic genes. Among the environmental variables, sample type (manure, soil, ditch water) explained most of the variance (Table 2 in publication **II**). Genes conferring resistance to aminoglycosides, disinfectants, MLSB, tetracyclines, sulphonamide, trimethoprim or vancomycin were common in manure and soils after manure application (Figure 3). These manure-associated genes were not detected in unfertilized soil or in ditch water sampled before soil fertilization (Figure 3). On the contrary, genes encoding β-

lactamases or multidrug-efflux pump proteins were mainly present in unfertilized soil or in ditch water and these ARGs were undetected in manure (Figure 3).

The ARG profiles of unfertilized soil and ditch water were similar to soil sampled from an Alaskan research station representing limited human impact (Wanget al., 2016). Genes encoding β-lactamases and multidrug resistance are commonly found even in environments with very limited human influence (Allen et al., 2008; Piddock, 2006). Soil is known to be an immense reservoir of antibiotic resistance genes (e.g. Wright, 2010), and agricultural practices are known to disseminate ARGs to the environment; however, the natural and intrinsic soil resistome is often excluded in studies targeting agroecosystems.

Genes from all MGE groups were present in all samples, while integrons, insertion sequences and transposases were more prevalent in manure than in unfertilized soil or in ditch water (Figure 3). On the contrary, plasmids were more abundant in ditch water and were also found in unfertilized soil (Figure 3). Natural fresh waters near agricultural fields are used for irrigation in many parts of the world during periods of inadequate rainfall, and thus bacteria carrying resistance genes might find their way from watercourses to the soil and forage crops. Mollenkopf et al. (2016) found a metallo-β-lactamase gene $bla_{\text{IMP-27}}$ on an IncQ1 plasmid from a swine farm in US and the plasmid-harboring $bla_{\text{IMP-27}}$ was carried by several bacterial species. In this study, bla_{IMP} genes and plasmid markers were co-detected in ditch water (Figure 3). This finding supports the hyopthesis that cycling of bacteria between the farm environment and farm animals, along with the use of selective antibiotics and the presence of MGEs, might be one cause for the transfer of resistance genes from environmental bacteria to human or animal associated bacteria. Indeed, clinically relevant β-lactamases might be incorporated in MGEs (Figure 4); however, most these genes were not detected or abundant in manure samples (Figure 3).

4.3 EFFECT OF MANURE FERTILIZATION ON ARGS IN FARM ENVIRONMENT (I, II)

The relative abundances of bla_{OXA58}, *sul1*, and *tetM* were elevated in soil immediately following manure application (Figure 1 in publication **I**). The roughly fourfold increase in the relative abundance of these genes was also statistically significant (Table 2). When examining the genes bla_{OXA58}, *sul1*, and *tetM* individually, the highest increase, over sevenfold, was found for *sul1*, whereas the abundance of *tetM* only exhibited a slightly over twofold increase (Table 2). Following land application, manure-associated ARGs and MGEs, which were not detected in soil before fertilization, were present in soil, demonstrating that they disseminated to soil as a consequence of manure application (Figure 3). Among the tested environmental variables, fertilization explained most of the variance in soil samples collected after fertilization (Table 2 in publication **II**).

Six genes that were detected in manure were found in ditch water sampled after manure application, implying potential dissemination from manure to ditch water (Figure 3). Four of these genes were MGEs (*intI2*, IS613, ISSm2 and *tnpA*/IS21) discovered on the farms T1 and T4 (Figure 3). However, the vast majority of genes

abundant in manure were not found in water, which means that the studied farms are probably not widely disseminating a large load of ARGs and MGEs further to the environment.

Despite dissemination to soil, the abundance and number of manure-associated ARGs and MGEs clearly decreased from samples taken immediately after land application to soil samples taken 2 and 6 weeks after fertilization (Figures 3 and 5). A more than fourfold decrease in the relative abundances of ARGs and MGEs was observed for 34 genes (Figure 5). The decrease was statistically significant for 30 genes, varying from 200- to 9000-fold (supporting information in publication **II**). The clear decrease in manure-associated genes over only six weeks suggests that manure-originating bacteria, which carry these genes, are not well adapted to the soil environment and/or that little to no horizontal transfer of ARGs occurs in manure-fertilized soil.

Table 2. Linear mixed models used in publication **I** and their results.

Model	Genes	Samples compared	Fixed effect	Random effects	Fold change (± SE)	*p*-value
Manure application†)	*bla*$_{OXA58}$	SB to S2WA	Before / after the application of manure	Sample type, Farm	5.55 (± 2.10)	2.77×10^{-2}
Manure application†)	*sul1*	SB to S2WA	Before / after the application of manure	Sample type, Farm	7.39 (± 1.98)	6.47×10^{-3}
Manure application†)	*tetM*	SB to S2WA	Before / after the application of manure	Sample type, Farm	2.2 (± 1.38)	1.89×10^{-2}
Manure application†)	*bla*$_{OXA58}$, *sul1*, *tetM*	SB to S2WA	Before / after the application of manure	Sample type, Farm, ARG	4.4 (± 1.41)	4.93×10^{-5}
Manure storage‡)	*bla*$_{OXA58}$, *sul1, tetM*	I to M	Fresh / stored manure	Farm, ARG	5.16 (± 1.54)	2.82×10^{-4}
Production animal‡)§)	*bla*$_{OXA58}$, *sul1*, *tetM*	I to I, M to M	Dairy cattle / swine	ARG	N.A.	>0.05

†) Farm T3 was not included in the manure application models for *bla*$_{OXA58}$
‡) Farm T3 was not included for *bla*$_{OXA58}$
§) *Post hoc* analysis

Notwithstanding the obvious decrease in the abundance of manure-associated ARGs in soil over time, a few of the MGEs and ARGs that disseminated to soil with manure were still elevated in soil 6 weeks after fertilization (Figure 5). Since these persisting ARGs and MGEs and *tetM* were not detected in unfertilized soil, the results suggest that ARG abundance, although elevated due to fertilization, decreases during winter. Thus, in Finnish farms, alterations in soil resistance profiles caused by annual manure fertilization are cyclical, depending on the year and time elapsed since manure application. Nõlvak et al. (2016) made a similar observation: the abundance of ARGs that disseminated to soil with manure decreased over time, although the abundance did not reach the background level during the time period in their study.

The winter, including e.g. freezing of topsoil for up to four months, might explain these findings, in part, although Finnish agricultural practices may also have a distinct impact. In Finland, manure application is only allowed from April-May to September-October. Thus, the soil does not receive manure dosages throughout the year but is left without land application from seven to ten months. In addition, the

legislation obliges farmers and veterinarians to prudent antibiotic use, which means that animals are treated individually and symptomatically, and are only medicated with antibiotics prescribed by veterinarians. Moreover, the most commonly used antibiotic in production animals in Finland is benzylpenicillin, which does not seem to disseminate resistance as vigorously as broad-spectrum antibiotics or antibiotic combinations. Resistance levels in bacteria found in food animals are generally lower in Nordic countries that use mostly benzylpenicillin for treatment compared with many other parts of Europe (European Medicines Agency, 2013; 2015). The current policy on antibiotic use and practices on manure application rates in Finland may lead to a low initial proportion of bacteria harboring resistance genes in manure compared to countries where antibiotics are used non-therapeutically or prophylactically and manure application rates are not controlled.

Schmitt et al. (2006) found that Swiss farmland that had previously been exposed to intensive farming and high manure loads harbored multiple resistance determinants and resistant bacteria even years after the most recent application of manure. In addition to exposure to manure of animals treated with antibiotics, fecal contamination has been linked to a higher prevalence of resistance genes in various environments (Muziasari et al., 2016; 2017; Onan and LaPara, 2003). Since the soils sampled before fertilization harbored a very low initial proportion of resistance genes related to fecal contamination, the results suggest that in addition to decreasing antibiotic use, reducing manure application rates and volumes might also help in reducing the dissemination of ARGs and MGEs to the farm environment. Unfortunately, there is a lack of studies from settings where antibiotic use is high and the influence of manure application on the soil resistome is followed over extended periods of time, and making it difficult to perform comparisons between different practices. Nevertheless, it has been found that high and unmonitored use of antibiotics on swine farms raises the diversity and abundance of the antibiotic resistance reservoir in the farm environment to an alarming level (Zhu et al, 2013).

4.3.1 EFFECT OF MANURE FERTILIZATION ON INTRINSICT SOIL RESISTOME

When manure is applied to soil harboring bacteria carrying e.g. β-lactamase genes, the manure may enrich these bacteria, resulting in an elevated abundance of β-lactamase genes (Hu et al., 2016; Udikovic-Kolic et al., 2014). If the abundance is increased during forage harvesting time, the bacteria might be transported to animals among the harvested crops. However, in this study, manure fertilization did not increase the abundance of genes found in unfertilized soil (Figure 3).

Samples taken from soil before annual fertilization in spring did not show high diversity of ARGs or MGEs, although these soils have been in agricultural use for several decades and received manure annually during growing seasons (Figure 3). Nevertheless, the resistance genes *sul1* and bla_{OXA58} could be detected in some unfertilized soil samples (Figure 1 in publication **I**). Soils in constant agricultural use might have a relatively stable population of resistant bacteria. Insertion sequences and class 1 integrons are considered to be markers of ARG contamination in the

environment (Gaze et al., 2011; Gillings et al., 2015). Intriguingly, insertion sequences or class 1 integrons were not found in unfertilized soil (Figure 3). Moreover, the ARG profiles of unfertilized soil samples were similar to soils that have not been in agricultural use (Wang et al., 2016).

4.4 CHANGES IN ARG ABUNDACE DURING MANURE STORAGE (I, II)

The ARGs *sul1* and *tetM* were found in both fresh and stored manure from all of the farms (Figure 1 in **I**). bla_{OXA58} was found in all of the manure samples except for farm T3 (Figure 1 in **I**). The ARGs detected in manure were significantly enriched, by roughly fivefold, in stored manure on all farms (Table 2). According to the LMM models, the production animal species did not affect relative ARG abundance in manure (Table 2). Instead, storing of manure was the only significant variable in the manure storage model (Table 2).

The increase in the relative abundance of ARGs during manure storage was confirmed for multiple genes in publication **II**: The relative abundance increased more than fourfold for 41 genes (Figure 5). The highest increase (up to 65-fold) was observed in the abundance of tetracycline resistance genes, followed by sulphonamide and aminoglycoside resistance genes with up to 45-fold and 41-fold increases in stored manure, respectively (supporting information in publication **II**). Altogether, ARG enrichment was statistically significant for 37 genes (supporting information in publication **II**). According to permutational multivariate analysis of variance (Table 2 in publication **II**), storing of manure explained 15 % of the variance in ARG and MGE abubdances. The number of animals and animal species explained most of the variance (25 % and 21 %, respectively). However, while the number of animals and animal species probably affect the diversity of ARGs and MGEs, these variables likely do not cause the enrichment of the genes in stored manure.

The increase in the relative abundance of ARGs in stored manure may arise as a result of a shift in bacterial population composition from the gastrointestinal tract community to a bacterial community that is better adapted to the conditions in the manure lagoon or storage silo. The conditions in the gastrointestinal tract and in a manure storage pit outside a piggery or cowhouse are very different, and previous reports show shifts in bacterial community from fresh to stored manure previously (Cotta et al., 2003). The change in community composition might thus explain the increased ARG abundance in stored manure seen in our results, assuming that the community that is better adapted to the conditions in manure pit is the community carrying these genes.

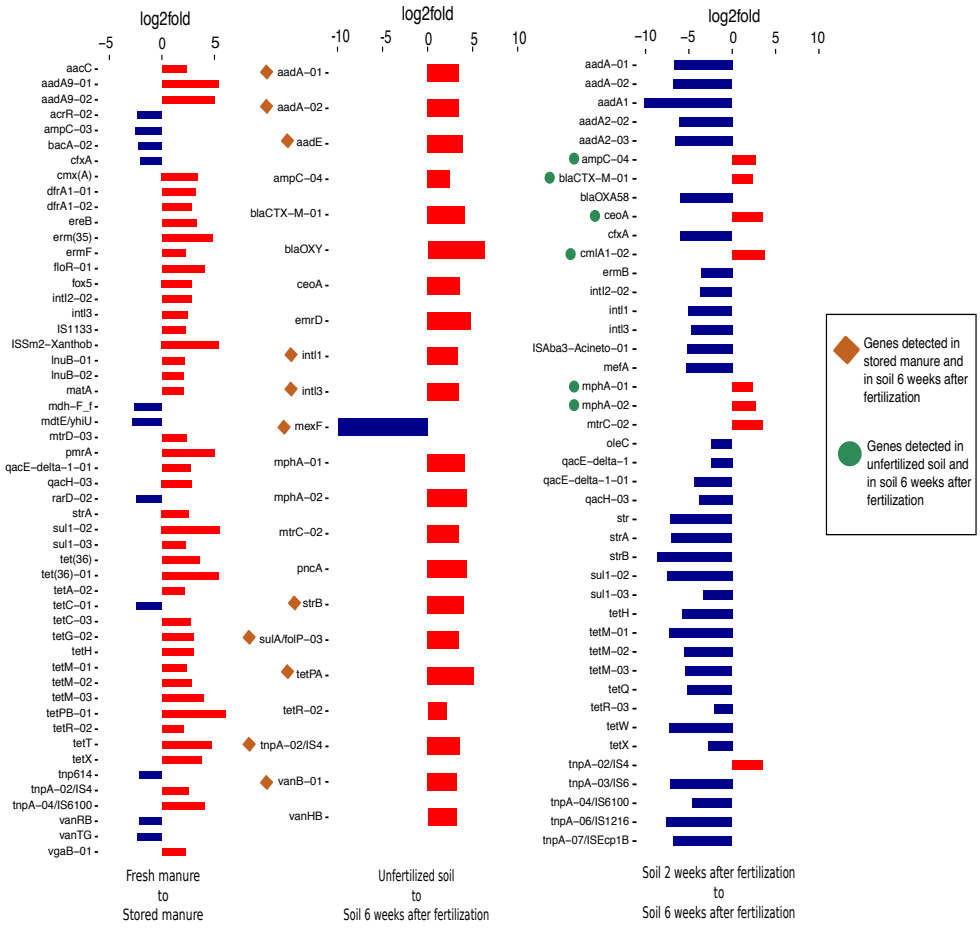


Figure 5. Fold change in the abundance of genes from fresh manure (I) to stored manure (M) (left panel), unfertilized soil (SB) to soil 6 weeks after fertilization (S6WA) (middle panel), and soil 2 weeks after fertilization (S2WA) to soil 6 weeks after fertilization (S6WA) (right panel). Fold changes are log2-transformed. Only genes with significant ($p < 0.05$) at least 4-fold increase or decrease are shown (Table S2). The genes that disseminated to soil with manure and persisted after 6 weeks are marked with a brown square, and genes that were detected in unfertilized soil and were elevated in soil 6 weeks after fertilization with a green circle.

Trace concentrations of antibiotics could also explain the enrichment of certain genes in stored manure. However, antibiotics were not detected from manure in publication **I**. Nevertheless, during the time when manure was collected, some animals in the farms were medicated with antibiotics. As there was a constant supply of fresh manure to the manure storage pit, small concentrations of antibiotics might have been transported along with manure to torage, as a considerable part of the antibiotics administered to animals are secreted in urine (e.g. Boothe 2016; Prescott, 2000). Therefore, traces of antibiotics below detection limit concentrations might have had an effect on ARG abundances in stored manure. Previously, it has been shown that transformation of resistance genes could be fostered by low antibiotic concentrations (Prudhomme et al., 2006). Moreover, as a response to subinhibitory concentrations of antibiotics, bacteria can increase genetic material exchange rates, promoting horizontal dissemination of resistance genes, though the SOS response mechanism

(Andersson and Hughes, 2014; Beaber et al., 2004; Davies et al., 2006; Guerin et al., 2009).

In addition to a shift in community composition, an increase in the relative abundance of ARGs in stored manure may arise due to induced HGT, since many of the enriched ARGs might be integrated in MGEs (Figure 4). MGEs co-occurring with enriched ARGs have been linked to these ARGs in various studies: For example, parts of the conserved regions of class 1 integrons, *qacE*Δ*1*, *sul1* and *intI*, were co-enriched along with the aminoglycoside resistane gene *aadA* that is commonly found in the gene cassettes of class 1 integrons (Guerra et al., 2000; Paulsen et al., 1993; Werner et al., 2001). Most tetracycline genes were also enriched in stored manure, and insertion sequences harboring transposases have been shown to accompany these genes (Götz et al., 1996; Jones et al., 2006; Li et al., 2011). Moreover, conjugative transposons have been shown to carry aminoglycoside, macrolide and tetracycline resistance genes (Bryan et al., 2004; Cochetti et al., 2008; Roberts and Mullany, 2011; Salyers et al., 1995).

The change in environmental conditions from the animal intestine to the storage pit could also increase the abundance of ARGs by causing induction in HGT rates through stress-response mechanisms (Poole, 2012). Miller et al. (2014) noticed that *sul1* and *intI* were co-enriched in wastewater sludge, which was stored at cold temperatures. The authors suggest that the enrichment might have arisen due to induced HGT caused by SOS response mechanisms, possibly triggered by cold stress (Miller et al., 2014). During winter, the temperature in manure in the storage silo or lagoon can be below 5°C in a boreal climate. Therefore, cold stress, trace concentrations of antibiotics and community shifts could all combine to cause ARG enrichment during the period of manure storage.

4.5 FIELD SUITABLE SCREENING PROTOCOL FOR ANTIBIOTIC CONTAMINATION (III)

Modern intensive farming and rampant use of antibiotics also disseminate antibiotic contamination to the farm environment in countries where antibiotic use is high and unrestricted. Tetracycline pollution has been detected in agricultural environments (Campagnolo et al., 2002; Chee-Sanford et al., 2009) and even in foodstuffs of animal origin (Cháfer-Pericás et al., 2010). Trace concentrations of tetracycline should be monitored due to their capability to disseminate antibiotic resistance (Fitzgerald, 2012; Price et al., 2012), toxicity to environmental organisms (Halling-Sorensen et al., 2002) and adverse human health effects caused by intake of contaminated food products (Sánchez et al., 2004).

Tetracyclines have three different acid dissociation constant (p*K*a) values, and they may exist as cationic, zwitterionic, or anionic species. Owing to this feature, tetracyclines may become sorbed to soil by various mechanisms, depending e.g. on ethe pH of the soil (Sassman and Lee, 2005). A common procedure for detection of contaminants, including in soil, involves extraction of compounds of interest from the sample using solvents, followed by quantification of the concentrations of the compounds in question from the extract solution. Unfortunately, there is a risk that the

extraction procedure may cause degradation of the compounds of interest leading to false negative results (Wegst-Uhrich et al., 2014). Moreover, the extracted concentrations measured by instruments do not necessarily correspond to the concentrations responsible for biological effects (Aga et al., 2016; Song et al, 2017).

The screening protocol established in publication **III** is a robust and inexpensive alternative to quantification of tetracycline. The protocol utilizes a recombinant luminescent *E. coli* K-12 sensor strain (Korpela et al., 1998) and commercially available polyester swabs, which are commonly used in the electronics industry. With tetracycline bioreporter cells, tetracycline detection is based on a naturally occurring tetracycline resistance mechanism (Figure 2) (Korpela et al., 1998). Furthermore, quantification of tetracycline is possible in field conditions without pipetting or extraction. Although pipetting is a simple task in the laboratory, it is a skill that requires training and is difficult to conduct precisely in the field. With the protocol, researchers could identify those samples in the field, which should be taken to the laboratory for further analysis. The simplicity of the protocol would allow for the utilization of citizen science volunteers for conducting field measurements to monitor compounds possibly indicative of emerging threats (Cohn, 2008; Silvertown, 2009).

In the experiments performed, the induction coefficient values calculated from Milli-Q water, milk and soil test samples with 6 tetracycline concentrations (0, 5, 15, 25, 50, 75 & 100 ng ml^{-1}) increased with increasing tetracycline concentrations in measurements by both luminometers used (Figures 4 and 5 in publication **III**). The peak of induction was achieved at the tetracycline concentration 50 ng ml^{-1} in Milli-Q water test samples (Figure 4. in publication **III**), which is in line with previous characterization of the biosensor (Korpela et al., 1998).

In order to be suited for reliable screening, reproducibility of the protocol is important. Concerning this, Milli-Q water, milk and soil test samples containing 100 ng ml^{-1} tetracycline were clearly distinguished from respective 0 ng ml^{-1} tetracycline test samples (Table 3). The standard deviations of 10 replicate swab assays from Milli-Q water, milk and soil test samples with 0 ng ml^{-1} and 100 ng ml^{-1} tetracycline varied from 4 % to 19 % (Table 3), which is in the same range as expected with manual pipetting (Pandya et al., 2010). The standard deviations remained below 10 % in Milli-Q water test samples in both concentrations and incubation time points (Table 3), and the protocol would therefore be best suited for detection of tetracycline contamination in water samples, for example, rivers near pharmaceutical companies in developing countries, as described by Larsson (2014). Standard deviations in soil test samples varied between 12 % and 17 % (Table 3) despite the fact that soil is a complicated matrix.

Milk test samples had the highest standard deviations and the highest luminescence values, ranging between 0.46 RLU (0 ng ml^{-1} tetracycline, incubated for 120 min) and 18.3 RLU (100 ng ml^{-1} tetracycline, incubated for 180 min) (Table 3). The high luminescence and standard deviation values compared to other samples were probably caused by additional nutrients in milk and matrix-effects, respectively. The antimicrobial efficiency of tetracycline is known to be dependent on the concentration of divalent cations capable of chelating the tetracycline molecule (Boothe, 2016; Korpela et al., 1998; Prescott, 2000).

Table 3. Mean luminescence values as relative light units (RLU) and standard deviations (S.D.) of ten replicate swabs from control samples and from 100 ng ml^{-1} tetracycline test samples. Luminescence was measured with a portable luminometer (BioOrbit 1253) after 2 and 3 hours of incubation.

	120 min incubation				**180 min incubation**			
	Tetracycline concentration ng ml^{-1}							
	0		100		0		100	
	Luminescence (RLU)	**S.D. (%)**	**Luminescence (RLU)**	**S.D. (%)**	**Luminescence (RLU)**	**S.D. (%)**	**Luminescence (RLU)**	**S.D. (%)**
Milli-Q water	0.162	9	9.826	5	0.220	4	11.112	7
Milk	0.455	11	16.15	16	0.921	16	18.315	19
Soil	0.100	17	1.865	14	0.191	12	1.594	12

As expected, soil test samples had the lowest RLU values. However, RLU values were still almost twenty-times higher in 100 ng ml^{-1} tetracycline swabs compared to 0 ng ml^{-1} tetracycline swabs after 120 minutes of incubation (Table 3). This demonstrates that despite the quenching of the light signal caused by light-absorbing soil particles, the protocol can potentially be further developed such that soils contaminated with tetracycline residues can be identified already at the field.

In further development of the protocol, quenching of the signal caused by sample matrix could be taken into account by using reference materials (Mackey et al., 2010; Sharpless et al., 2015) to produce field standards, such that the matrixes of studied samples and the standard with a known tetracycline concentration closely resemble each other. Another approach would be to use a non-specific control strain that constitutively produces luminescence (Ivask et al., 2001; Peltola et al., 2005) in addition to the reporter strain. The use of a control strain could be used to control for the complexity of environmental samples (Ivask et al., 2009; Poulsen et al., 2006).

The swab assay presented allows for rapid and cost-efficient screening of environmental samples and foodstuffs for tetracycline traces. The choice of a luminometer device used for measuring the luminescence did not affect the results, and therefore, swabs can be measured either in the field with a portable luminometer or swabs dipped in samples in the field could be transported to a laboratory where luminescence can be measured using a plate reader. Swabs can be used to replace chemical extraction of antibiotic traces. Therefore, combining swabs with biosensors could help in progressing from mere detection of antibiotic contaminants to the evaluation of their ecological effects in the studied environment.

5 CONCLUSIONS AND FUTURE PROSPECTS

In this study, I showed that despite restricted antibiotic use in Finland, resistance determinants in manure disseminated to agroecosystems. The dissemination was linked to enrichment during winter storage of manure, which is a necessary stage in the boreal climate. However, it was evident that by harvesting time most of the disseminated genes had decreased to a level below the detection limit in soil. Moreover, the few genes that persisted most likely decayed during winter, since they were not found in soil before annual manure fertilization. My conclusion is that this reduction is partially associated with Finnish agricultural practices that limit manure application and with restricted antibiotic use policy, but also freezing of the topsoil during winter probably reduces the abundance of these genes.

Despite the fact that these observations are favorable compared to the global situation, even Finnish agroecosystems might pose a threat, since emerging resistance genes were found in unfertilized soil and ditch water. Due to the circulation of resistance determinants in production animal farms, agroecosystems might represent a link between the environmental resistome and resistant clinical pathogens. Thus, if manure application rates and volumes were increased, or if broad-spectrum antibiotics were used for medicating animals, environmental resistance genes could be selected in the animal gut microbiome.

The innovation of utilizing swabs together with bioreporters is a cost-efficient approach to screening for antibiotic contamination. It may also help in evaluation of the ecological effects of antibiotic traces. More information regarding these issues is required, especially from rivers next to pharmaceutical companies and large livestock feedlots in countries where antibiotic use is unrestricted.

I filled some important knowledge gaps concerning the effects of animal husbandry under limited antibiotic use. However, many questions still remain. Clarifying the reason for the enrichment of resistance genes and mobile genetic elements during winter storage of manure is critical, since storing biological waste materials in order to recover their nutrients is becoming increasingly common due to threatening phosphorus depletion. Because of ARG enrichment, a larger load of resistance determinants might find their way to agroecosystems where they may spread further through, for example, leachate water, dust, flies and birds. Another important question concerns the identity of the bacteria carrying the intrinsic resistance genes found in the farm environment, and the factors that account for their increased abundance. Some of the remaining questions could be answered with a combination of culture-independent methods and sampling strategies tailored to provide information about the effect of agricultural practices.

This thesis forms a comprehensive work on antibiotic resistance in agroecosystems, designed to reveal the role of agricultural practices. Antibiotic resistance will remain a global problem in the foreseeable future, and we desperately need to develop a comprehensive understanding on the phenomenon. The promotion of nutrient recycling will make this understanding even more crucial in the future.

ACKNOWLEDGEMENTS

This work was carried out at the Division of Microbiology and Biotechnology, Department of Food and Environmental Sciences, University of Helsinki. The work was supported by Maj and Tor Nessling foundation, Academy of Finland, EnSTe graduate school and MBDP graduate school. Seivästö foundation is acknowledged for grant for finalizing this thesis. I am thankful to all my supporters.

I am truly grateful to my supervisors Professor Marko Virta and Professor Markku Yli-Halla, I have learned a lot from you and have enjoyed our conversations, in both science and life. Marko, I want to thank you for that you have trusted in me by giving me freedom to do my work relatively independently, given me many great opportunities to present my work and encouraged me to always try my best. Markku, I owe my deepest gratitude to you for teaching me the fundamentals of science and for acting my mentor.

Jenni, I'm not sure if I would have made it here without you. I cannot thank you enough! You will always be *the* soil science professor form me. Christina, thank you for all your help and that you have always believed in me. Johannes, thank you for your friendship and correcting the language of this thesis, I highly appreciated your preciseness. Kata, thank you for all your contributions and always answering my questions. Windi, I think I will live several years longer because you have made me laugh so often, thank you for doing that! Matti, I want to thank you for your contribution but also for our discussions related to statistics, R and life. Avinash, thank you for precise lab work. I am thankful to everybody who is or has been a member of *MMX-family, our office* or *our coffee table*; you have made this easier for me in so many ways. I want to express my gratitude also to the personnel in MIB-division, especially Mika, Ritu and Pekka, and for Hannele T all the special help.

I want to also thank all my co-authors, particularly Professor James Tiedje and Dr. Bob Stedtfeldt from Michigan State University; I really appreciated the time you used in improving our paper. I'm also grateful for all the people hosting me during my scientific visits in the U.S. and Canada. Dr. Jean McLain, thank you for ARASOS.

I am extremely thankful to farms T1, T2, T3, and T4 for making all this possible and to veterinarians at the Department of Production Animal Medicine at the Saari unit for help. I am particularly thankful to Professor (Emerita) Satu Pyörälä for explaining the historical events related to antibiotic use in Finland and her patience with me.

I'm very grateful to Professor Jaak Truu for his willingness to act as my opponent and pleased for having professor Kaarina Sivonen as my custos, thank you for pushing me forward! I feel fortunate that I had two excellent reviewers, Dr. Eddie Cytryn and Docent Petri Auvinen, thank you for your excellent comments.

I would like to offer my special thanks to everybody involved in One Health Finland, my colleagues at Division of Environmental Soil Science and Faculty of Veterinary Medicine. Professor Raimo and Dr. Jere, I would not be here without you. Thank you also to my teachers at Porvoo Vocational College, and to my current colleagues at Finnish Food Safety Authority Evira for tolerating me lately!

Very special thank you goes to my friends and horses; you and the life outside the university has helped me over many rough times.

I feel privileged that I have loving parents who have always supported me in everything I do. Without you I wouldn't have made it.

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